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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) Parkinson's disease (PD) is caused by deterioration of the dopamine (DA) nigrostriatal system. Loss of DA can be induced experimentally by neurotoxin lesion of DA neurotransmitter producing neurons in the substantia nigra, or through lesioning DA axon terminals in the striatum with subsequent degeneration of their cell bodies in the substantia nigra. We have produced and characterized a new animal model of preclinical PD. Experimental PD has been induced by unilateral, intranigral infusions of the neurotoxin malonate to produce partial loss of nigrostriatal DA. The animals were assessed weekly for forelimb asymmetries for 4 weeks to obtain a behavioral index of the subtotal DA loss. The striata were examined morphologically and compared to the intact, contralateral side. Presynaptic striatal DA terminal losses were determined by evaluation of expression of tyrosine hydroxylase. Differential changes in postsynaptic striatal DA receptor expression and cleaved caspase-3 occurred at 4 weeks after neurotoxin exposure. Subsequent experiments examined effects of a secondary stressor on further exacerbation of striatal changes following the neurotoxin lesion. Neurochemical analysis of residual striatal DA following neurotoxin and secondary stressor exposure was performed in parallel, using HPLC of DA and its metabolites. Some tissues are awaiting evaluation.				
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Introduction

This document covers research performed during July 1, 2001 through June 30, 2002. The intent of the research program has been to establish a preclinical rat model of Parkinson's disease (PD) to examine elements of the nigrostriatal system that are particularly susceptible to the early changes in dopamine (DA) levels. We have employed two different infusion sites and two different categories of neurotoxins to produce sub-total losses in DA: 1) intrastriatal 6-hydroxydopamine (6-OHDA) and 2) intranigral malonate. The objective was to infuse a quantity of neurotoxin that would yield a ~50% loss in striatal DA at 4 weeks after the introduction of the neurotoxin. Studies in the initial year of the research program showed that 6 µgm of intrastriatal 6-OHDA (in 2 µl ascorbate) would produce this level of DA loss. Experiments in the current year were directed at evaluating the dose of intranigral malonate needed to achieve this same level of DA loss as the prior 6-OHDA studies. Further experiments introduced a secondary stressor to the preclinical PD rat model, to assess whether subsequent changes could be induced in the nigrostriatal pathway. We employed a novel, species relevant stressor, TMT (2,5-dihydro-2,4,5-trimethylthiazoline) a synthetic product that replicates the active component of red fox feces, and is employed by the pest control industry (Pherotech, Inc., Delta BC, Canada).

The SOW for the grant was modified in the 01 year to produce a unilateral 50% DA lesion rat model, as our initial use of bilateral DA depletions resulted in a loss of robust, healthy rats and required excessive animal husbandry. We subsequently modified that SOW in the current year (3/28/02) to extend the end point of the research plan, without further financial obligation from the Department of Defense. This approved no-cost extension (5/20/02) of the assistance agreement is included in the appendix to this annual report.

Other personnel issues impacted on the performance of the research plan in the current year. 1) The research assistant (Agron B. Elezi) hired August 2001 left to attend medical school June 2002. A. Elezi had performed the cryostat sectioning of tissues, immunohistochemical staining for the DA receptors and cyclic AMP, and had learned to perform the HPLC analysis of DA and its metabolites on rat tissues prepared for the research project. 2) A second year medical school student (F. Craig Littlejohn) participated in a neuroscience elective for 3 months and determined the expression of striatal cleaved caspase-3 using animals prepared in this reporting period. 3) A first year medical school student (Thomas Buchanan) initiated a behavioral component on the evaluation of limb asymmetry for the lesioned animals that were prepared in this reporting period. T. Buchanan also began a morphological component to examine the expression of cleaved caspase-3 in identified striatopallidal neurons in the various neurotoxin lesioned paradigms. 4) A first year MD/PhD student (Jillian Theobald) participated in a month-long lab rotation to examine whether specific post-translational modification (e.g., site-specific cyclic AMP-mediated protein kinase A phosphorylation) of the striatal NR1 NMDA receptor subunit occurred following subtotal loss of DA in the rats behaviorally evaluated by T. Buchanan. 5) A college senior student intern (Anne E. Grissell) has initiated an independent study project to determine the cellular mechanism underlying the induction of cleaved caspase-3 levels in the partially DA deafferented striatum of the animals that have been behaviorally evaluated. 6) The co-PI for the project (Dr. Kathy Steece-Collier) left the Chicago Medical School on April 15, 2002 to take a position in Neurological Sciences at Rush-St. Luke's Presbyterian Medical Center in Chicago.

Another incident that severely impacted the productivity of the 03 year, was the loss of brain tissue samples due to backup generator failure on the electrical circuit supplying the main freezer

for the laboratory. We have repeated the preparation of some of these samples in addition to completing the proposed research for this year. This is detailed in the modified SOW request in the appendix section of the report.

Body

The modified SOW for the 03 year of this research project contains 3 stated aims:

1. Rats will be lesioned unilaterally using intranigral malonate infusion that provides ~50% loss in striatal DA on the ipsilateral side at a survival time of 4 weeks. A second series of rats will be produced with the same DA loss and then subjected to a secondary stressor event, e.g., the TMT stressor regimen, for an additional 4-5 weeks.
2. Half of the rats in prepared in #1 will be analyzed by HPLC to determine the striatal levels of DA and its metabolite DOPAC, to establish the appropriate dose of intranigral malonate to yield the criterion of ~50% striatal DA loss at 4 weeks. The change(s) in nigrostriatal DA produced by the secondary TMT stressor regimen will be assessed by HPLC.
3. The other half of the rats (produced in #1) will be analyzed by immunofluorescence histochemistry to establish striatal DA levels (using tyrosine hydroxylase [TH] immunohistochemistry), DA receptor distributions, and other postsynaptic striatal morphological indices. The data will be analyzed by cellular luminosity histogram values and conjoined with results from HPLC and behavior with and without the TMT secondary stressor treatment exposure.

Table I. Animals Prepared for Analysis in this Year of the Research Plan

<i>Experiment</i>	<i>Date</i>	<i>Survival</i>	<i>Count</i>	<i>Analysis</i>
<i>Intrastriatal 6-OHDA + TMT</i>	7/13/01	4 weeks + 5 weeks	8	HPLC (Yale)
<i>Intrastriatal 6-OHDA</i>	7/13/01	4 weeks + 5 weeks	5	HPLC (Yale)
<i>Intrastriatal 6-OHDA + TMT</i>	7/13/01	4 weeks + 5 weeks	8	IHF ¹
<i>Intrastriatal 6-OHDA</i>	7/13/01	4 weeks + 5 weeks	4	IHF ¹
<i>Intrastriatal 6-OHDA</i>	7/13/01	4 weeks	8	IHF ¹
<i>Intranigral malonate, 1 μmol</i>	12/17/01	4 weeks	6	HPLC
<i>Intranigral malonate, 2 μmol</i>	12/17/01	4 weeks	6	HPLC
<i>Intranigral malonate, 4 μmol</i>	12/17/01	4 weeks	6	HPLC
<i>Intranigral malonate, 1 μmol</i>	12/17/01	4 weeks	2	IHF
<i>Intranigral malonate, 2 μmol</i>	12/17/01	4 weeks	2	IHF
<i>Intranigral malonate, 4 μmol</i>	12/17/01	4 weeks	2	IHF
<i>Unilateral Nigral 6-OHDA</i>	1/9/02	1 week	4	IHF
<i>Unilateral Striatal 6-OHDA</i>	1/9/02	1 week	4	IHF
<i>Unilateral Nigral 6-OHDA</i>	2/13/02	4 weeks	6	IHF
<i>Unilateral Striatal ascorbate</i>	2/13/02	4 weeks	2	IHF
<i>Intranigral malonate, 3 μmol</i>	3/27/02	4 weeks	14	HPLC
<i>Intranigral malonate, 3 μmol</i>	3/27/02	4 weeks	14	Behavior ² and IHF
<i>Intrastriatal 6-OHDA + TMT</i>	6/5/02	4 weeks + 5 weeks	4	Behavior ³ and IHF
<i>Intranigral 6-OHDA + TMT</i>	6/5/02	4 weeks + 5 weeks	5	Behavior ³ and IHF

¹These samples were lost when the freezer thawed, 12/01.

²Nine and ³four animals were followed for limb asymmetry behavior (Tillerson et al, 2000).

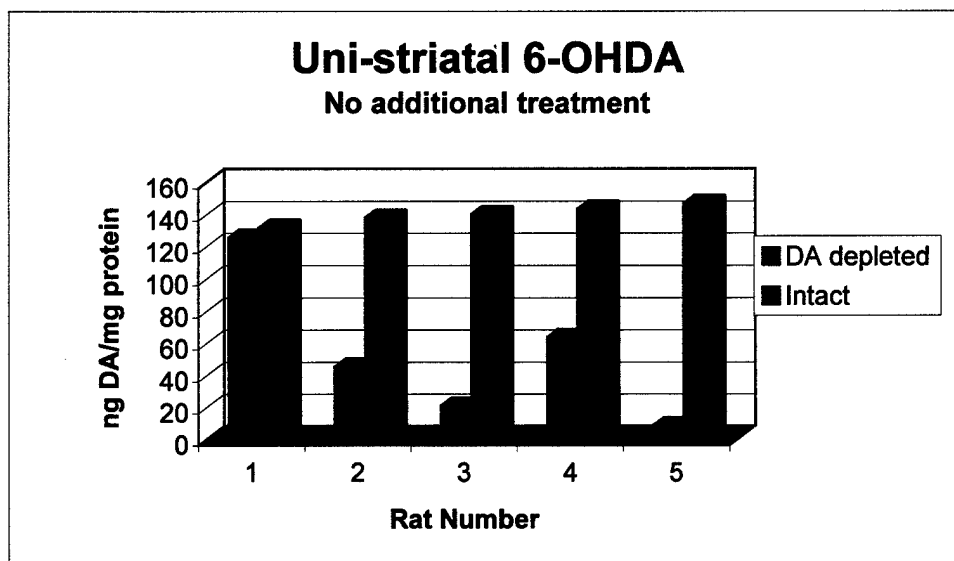
The experimental chronology begins with studies that had been planned and then initiated in the 02 year of the research plan, using a species-relevant secondary stressor on unilateral 6-OHDA intrastriatal lesioned rats. The 03 year studies are focused primarily on the outcome of studies associated with intranigral malonate infusions. These will be described in turn.

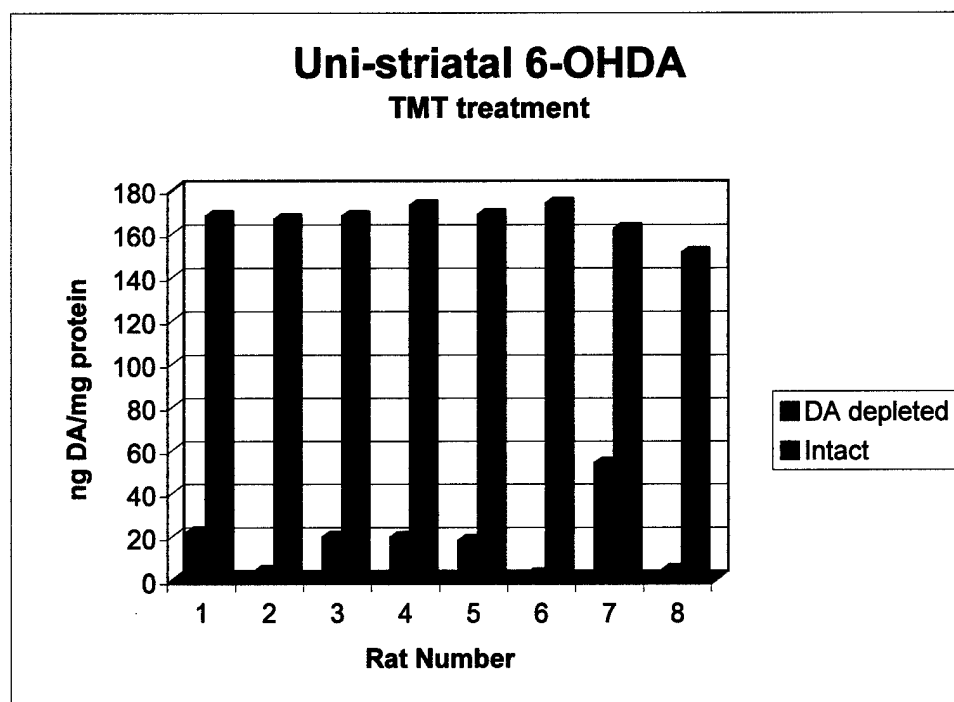
TMT Secondary Stressor Event

In our initial TMT regimen, preclinical PD animals were produced using intrastriatal 6-OHDA (6 μ gm in 2 μ l ascorbate), and "rested" for four 4 weeks. Rats were divided into TMT-exposed (N = 16) and control (N = 9) groups. TMT (30 μ l volume) was placed on filter paper circles, positioned on top of the wire cage lid, and the compound was allowed to volatilize for 1 hour in an isolation room. This strategy allowed the TMT-treated rats to associate the room change and presence of the filter paper with the impending exposure to the stress producing odorant. Rats exhibited typical fear reactions (Weninger et al, 1999) to the TMT scent, which consisted of burrowing in their cage bedding, staying at the opposite end of the cage, excessive oral and whisker grooming, and heightened motor activity, as described previously (Wallace and Rosen, 2001). Animals remained in the isolation room until the cages were cleaned on the following day with replacement of clean bedding, water and food pellets, then they were returned to their home animal room. TMT was presented once per week to assure that the animals did not habituate the experience. Animals were sacrificed after five weeks, and half of the brains were taken for HPLC analysis of the striatal DA and DOPAC levels. Our collaborator at Yale University, Dr. John Elsworth, performed the HPLC analysis of these samples.

Figure 1. Striatal DA was measured by HPLC, as described in Morrow (et al, 2000). (A) The loss of striatal DA, 9-weeks following intrastriatal infusion of 6 μ gm 6-OHDA (2 μ l in ascorbate) is shown. The average striatal DA loss in ng/mg protein \pm sem for the control sides was 140.22 ± 2.6 versus 6-OHDA lesioned sides 53.14 ± 20.5 ($p = 0.003$). (NOTE: Rat #1 did not have a successful 6-OHDA intrastriatal lesion, but was still included in the data set). (B) Exposure to the predator odor, TMT for 1 hour/week for 5 weeks produced a significant additional loss of striatal DA, 17.83 ± 0.85 (lesioned sides) versus 166.21 ± 2.5 (control sides), $p < 0.001$. Thus, an additional 90% loss of DA occurred as a consequence of TMT exposure and the continued deterioration of the nigrostriatal pathway after the 6-OHDA DA lesion.

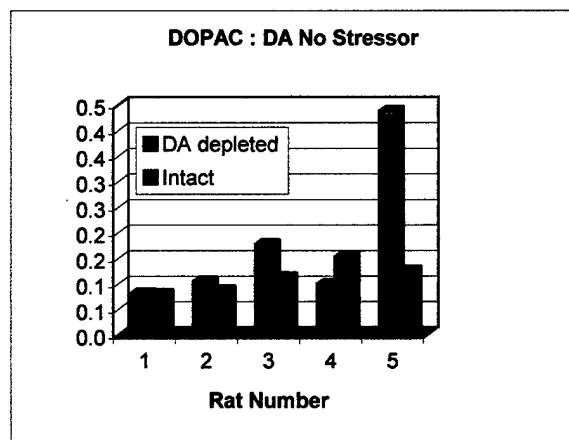
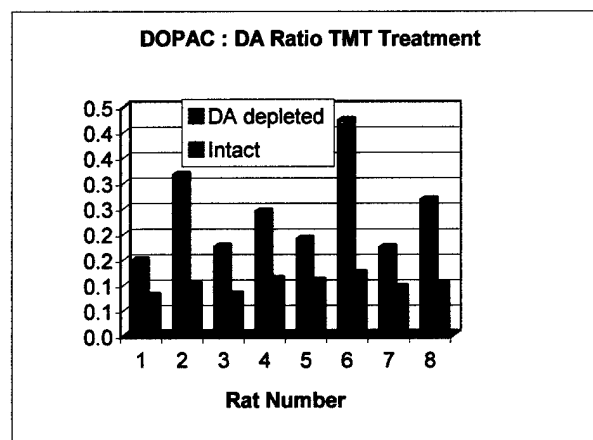
A



B

In addition, the primary DA metabolite, DOPAC was measured to obtain the ratio of DA turnover induced within the striatum by the 5-week TMT exposure regimen (reported as DOPAC:DA ratio). These data are shown in figure 2.

Figure 2. The striatal DOPAC:DA ratio for the control (A) and TMT treated (B) groups, 9 weeks after DA depletion due to unilateral 6 μ m intrastratial 6-OHDA. The average group values were **No Stressor:** $0.112 \pm .01$ intact versus $0.181 \pm .07$ DA depleted (no statistical difference); **TMT Treatment:** $0.099 \pm .005$ intact versus $0.242 \pm .033$ DA depleted ($p < 0.001$).

A**B**

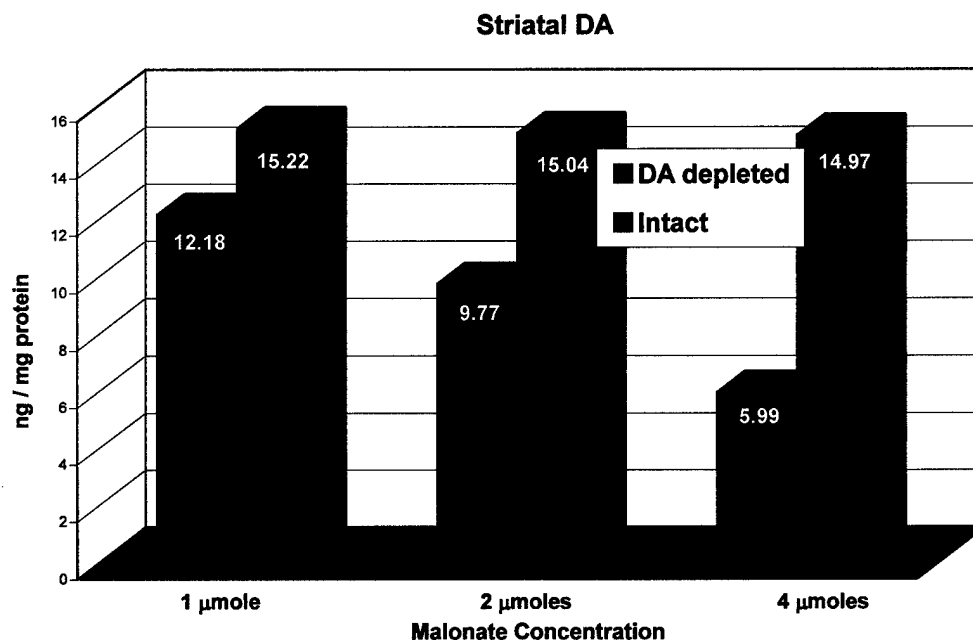
The other group of the TMT treated (N = 8) and control (N =4) samples were frozen for morphological evaluation of postsynaptic striatal expression of functional nigrostriatal indices (TH, DA receptor subtypes, cyclic AMP). Unfortunately, these tissues were stored in the -85°C freezer, and were destroyed when the power to the unit was interrupted (see appendix for details). These samples may still be viable for western analysis, and we will attempt this protocol in the upcoming grant period. We have performed an additional small pilot experiment to assess

whether there are any morphological correlates that underlie the HPLC and behavioral changes detected after the TMT regimen. In this pilot series of animals ($N = 9$), all rats were lesioned using 6-OHDA ($6 \mu\text{g}$), but the location of neurotoxin infusion was within either the striatum ($N = 4$) or the substantia nigra ($N = 5$) to evaluate the effect of neurotoxin placement. These rats were videotaped and scored to determine if limb asymmetries could be detected using the methods described by Dr. Tim Schallert and colleagues (Tillerson et al, 2001). All animals were subsequently exposed to 5 weeks of TMT exposure, using a 3 times per week bolus of $30 \mu\text{l}$ of TMT, as described above. Animals were killed 9 weeks following the 6-OHDA lesions, and these samples are stored at -86°C and awaiting morphological evaluation. The plan is to evaluate differences in the expression patterns for the DA receptor subtypes, the cyclic AMP second messenger, and to relate changes to specific projection pathways of the striatum, which demonstrate differential sensitivity to the loss of DA (Nisenbaum et al, 1999). Our novel observation of an elevation in the intensity and number of neurons expressing the activated form of caspase-3 in the DA depleted striatum will also be analyzed in this experimental group.

Malonate Neurotoxin Studies

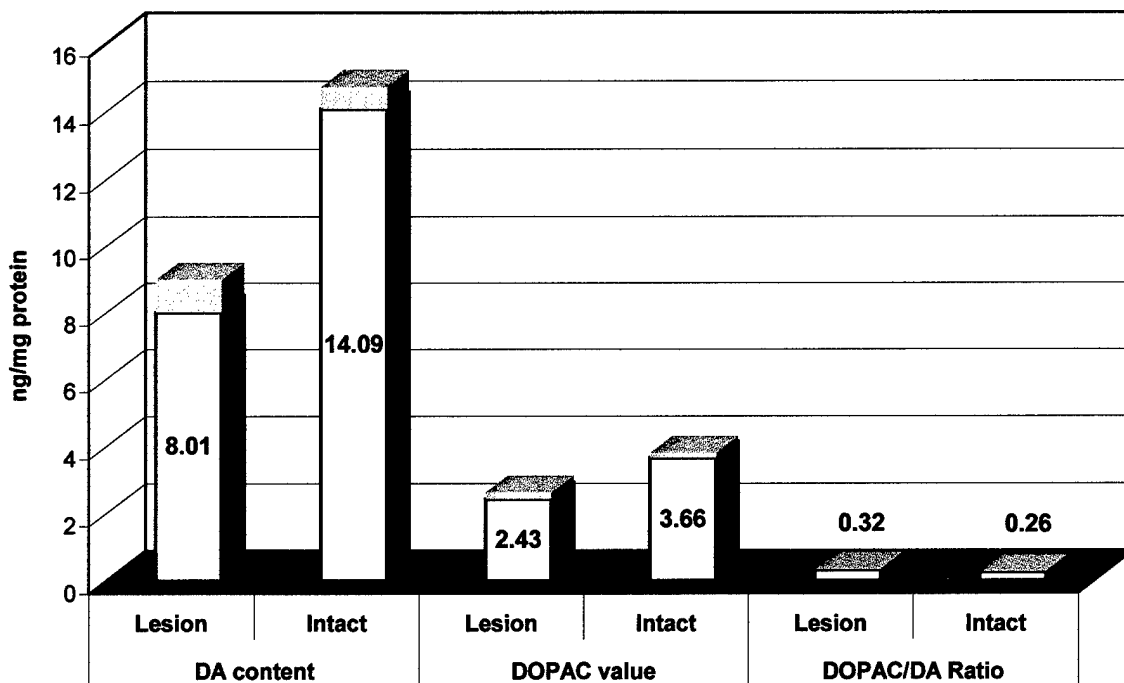
Our initial experiments using malonate were to establish the dose of intranigally infused neurotoxin that would yield $\sim 50\%$ loss of striatal DA at 4 weeks post-lesion, as stated in the SOW. We performed a series of infusions using 1, 2 and $4 \mu\text{moles}$ of malonate ($N = 8$ for each dosage), based upon the publications of Greenamyre, Sonsalla and their colleagues (Greene and Greenamyre, 1995; Albers et al, 1996). Animals were evaluated using HPLC ($N = 6$ each group) or analyzed using immunofluorescence histochemistry for TH, D2 DA subtype, cyclic AMP, cleaved caspase-3 versus NeuN or enkephalin ($N = 2$ each group).

Figure 3. HPLC analysis of DA was performed after variable malonate doses in $1 \mu\text{l}$ volume placed into the right substantia nigra. Values are the average of 6 animals at each dose, 4 weeks post-lesion. A. Elezi performed this analysis.



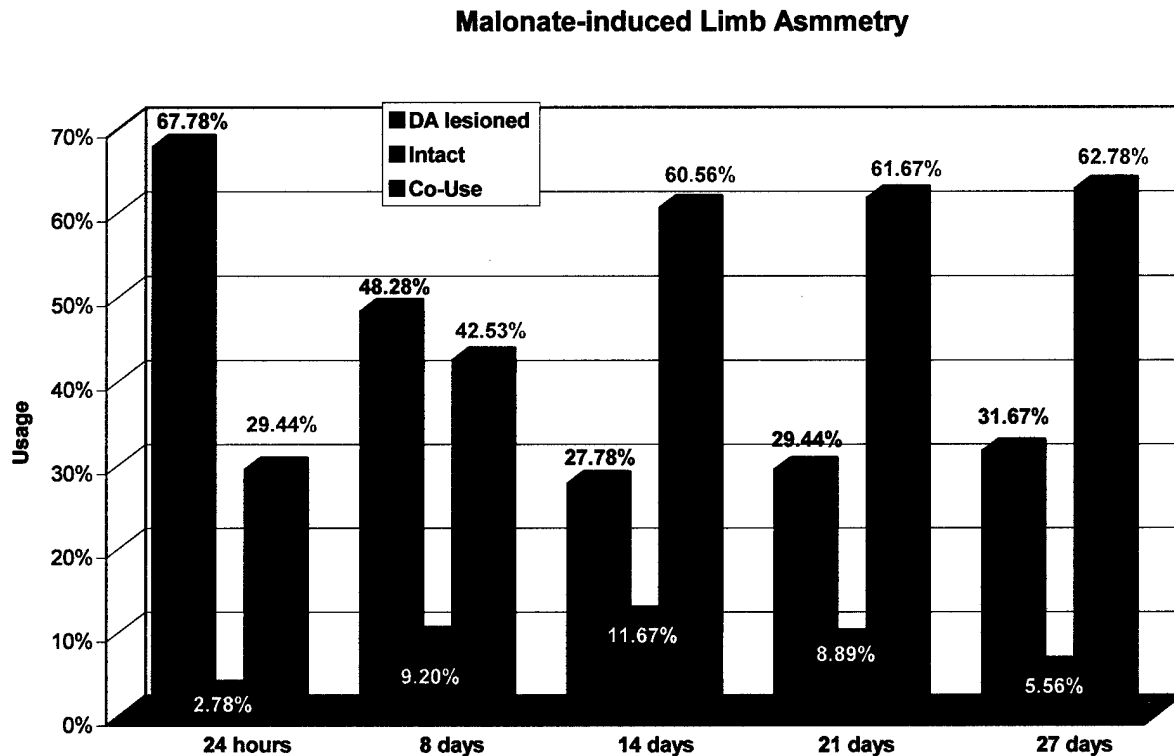
Two animals at each of the three different malonate doses were taken for immunohistochemical evaluation of striatal TH staining, cyclic AMP, DA receptor staining, and cleaved caspase-3 expression. The findings suggested that 3 μ moles malonate should be infused to mirror previous outcomes achieved using 6-OHDA. Thus, further experiments used this dose and duration for the malonate studies. We prepared a series of 28 animals using this dose of intranigral malonate and the animals survived for 4 weeks. Half of the animals were processed for HPLC analysis at sacrifice (figure 4), and 14 animals were frozen for immunofluorescence evaluation.

Figure 4. HPLC analysis was used to determine striatal DA and DOPAC:DA ratio in animals lesioned intranigrally with 3 μ mole malonate, 4 weeks following infusion of 1 μ l. Values are given as the average of N = 14 (white) with the sem shown above (blue) the appropriate column.



Of the 14 animals taken for morphological assessment, 9 were evaluated behaviorally using measurement of limb asymmetries (Tillerson et al, 2001). T. Buchanan performed this analysis of the animals once the dark cycle had ensued, once per week. Each rat was placed into a plexi-glass cylinder, which was narrow enough that the animal would rear up onto his hind paws to investigate the opening at the top. The task was to videotape usage of either the right or left forepaw or the use of both paws (co-use) to push around the circumference of the cylinder as this novel environment was explored. These data were scored for each rat as detailed in Tillerson (et al, 2001), and then plotted individually. While there were variations in the percent of usage between individual rats, the forepaw ipsilateral to the intranigral malonate infusion and striatal DA loss was employed more prevalently ($p < .05$) to push off from the vertical surface of the cylinder in the experimental group. The behavioral findings were averaged for all nine of the animals over the series of 4 weeks, and are plotted in figure 5. The morphological analysis of striatal neurochemical indices has just begun in these animals.

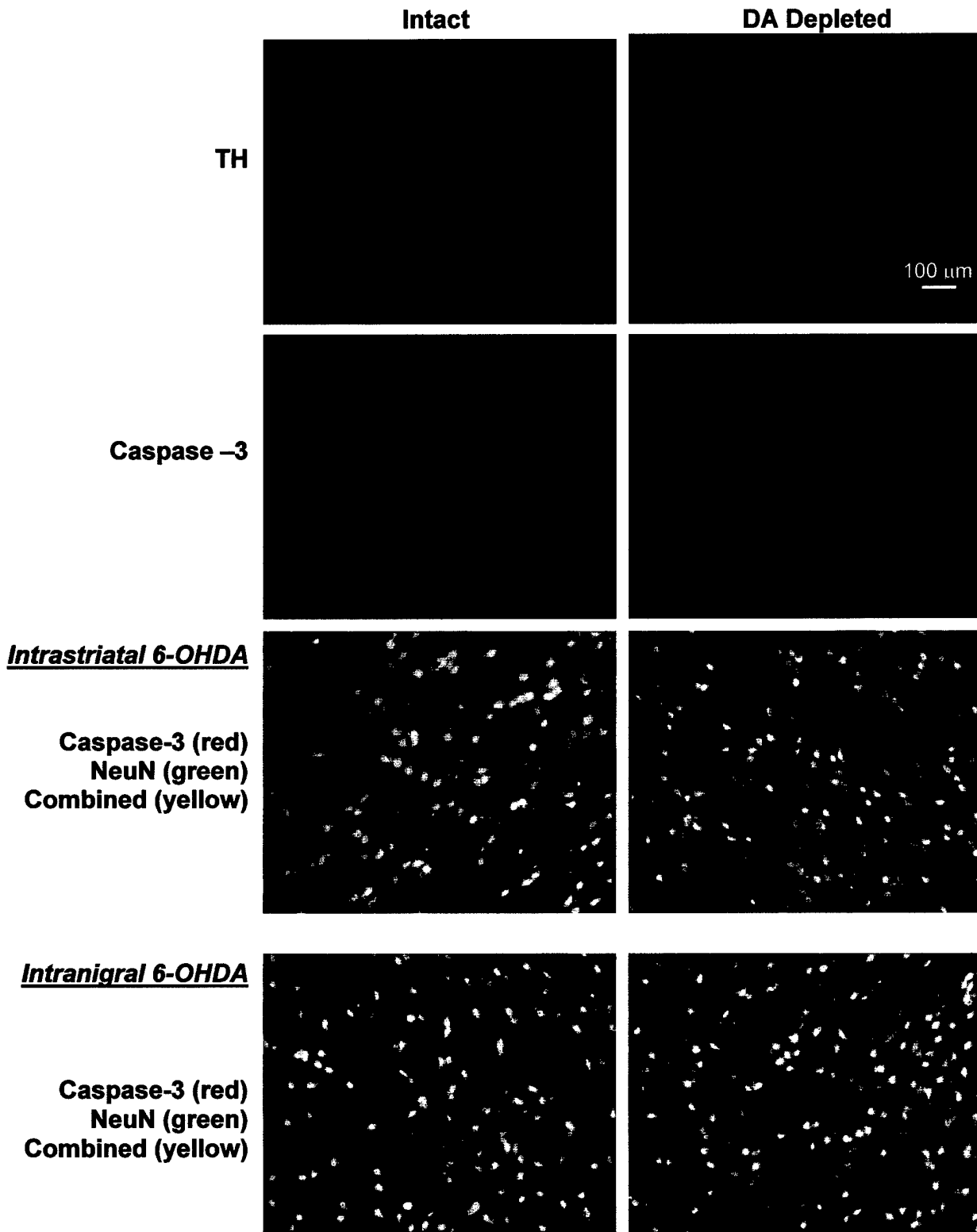
Figure 5. Behavioral measurements in the preclinical PD rats, lesioned by intranigral malonate ($3 \mu\text{mol}$ in $1 \mu\text{l}$), using the methods of Tillerson (et al, 2001) to score percent usage of forepaws in an exploratory task.



Morphological Evaluations

The past year of study demonstrated that only the D2 DA receptor subtype was consistently elevated in the DA deafferented striatum 4 weeks after the intrastriatal infusion of 6-OHDA. We also determined that a consistent elevation occurred in cyclic AMP in these same preclinical PD rats. We tested the supposition that striatal neurons might be destined for further deterioration due to the alteration in DA homeostasis, and examined this hypothesis by evaluation of the expression of cleaved caspase-3, a pivotal executioner enzyme in apoptosis, leading to subsequent neurodegeneration (Lu et al, 2000; 2001; Mattson, 2000). Our data demonstrated that cleaved caspase-3 was elevated within the DA-depleted striatum by 4 weeks following intrastriatal 6-OHDA neurotoxin infusion. The response was statistically significant, and some of the caspase-3 staining was detected specifically within neurons, as assessed by coincident staining with the neuron selective marker, NeuN. These data were analyzed using cellular luminosity histograms of random cells intersecting a counting grid, as described in Ariano, et al (2002). A representative set of experimental findings is shown in figure 6. Second-year medical school student, F. Craig Littlejohn, presented these studies as a poster in February 2002 at the Chicago Chapter meeting for the Society for Neuroscience. We concluded that cleaved (active) caspase-3 within striatal neurons was elevated by the loss of DA in both its cellular intensity of staining, and the number of reactive neurons. Regardless of whether the site of infusion was within the striatum or the substantia nigra, subtotal DA loss, induced by 6-OHDA infusion, produced neuronal elevations in striatal cleaved caspase-3 staining.

Figure 6. The expression of cleaved caspase-3 was enhanced significantly in staining intensity and in numbers of neurons within the striatum 4 weeks following DA loss, shown by the decreased TH luminosity (- 54% on lesioned side vs control value). The lesioned side is to the right of each panel, and data has been taken from two different animals.



Detection of immunofluorescent staining was performed using a digital camera, and the entire grayscale range from 0-255. A cell was considered a striatal neuron when located within the dorsal region, rostral to the decussation of the anterior commissure and was approximately 15 μm in diameter. A positive signal was established using a tissue section stained *without* primary antibody, processed concurrently. The fluorescence emitted from the striatal neuropil of the control section at the sampling magnification (20X) was assessed from 3 randomly chosen regions of interest. These values were subjected to a pixel-intensity measurement and the median value recorded for each sample area, then the values were averaged. This established the "background signal." Stained elements that were 20% brighter than this background signal were included in the data set. A counting grid was overlaid atop each image, and neurons meeting the above luminosity and size criteria, and intersecting the lines of the counting grid were recorded into the data set in Excel spreadsheets. Our counting grid has vertical lines spaced at 0.5-inch intervals across the width of the image taken with the 20X objective, and then viewed on the monitor at 100% size in Adobe Photoshop. Averages of the median luminosity values and standard error (sem) for neurons in each image from the control and DA depleted striata were computed. Two-tailed t-tests established statistical significance of the data set at $p < .001$. The overlap of signals, in the data shown in figure 6 for example, cleaved caspase-3 (red) expression within NeuN (green) neurons are visible as yellow cell bodies.

Figure 7. Cellular luminosity histograms were generated from the morphological data and demonstrated cleaved caspase-3 (red), NeuN (green), and neuronal caspase-3 (yellow) between the control and DA depleted striata. Asterisks denote statistical significance ($p < 0.001$).

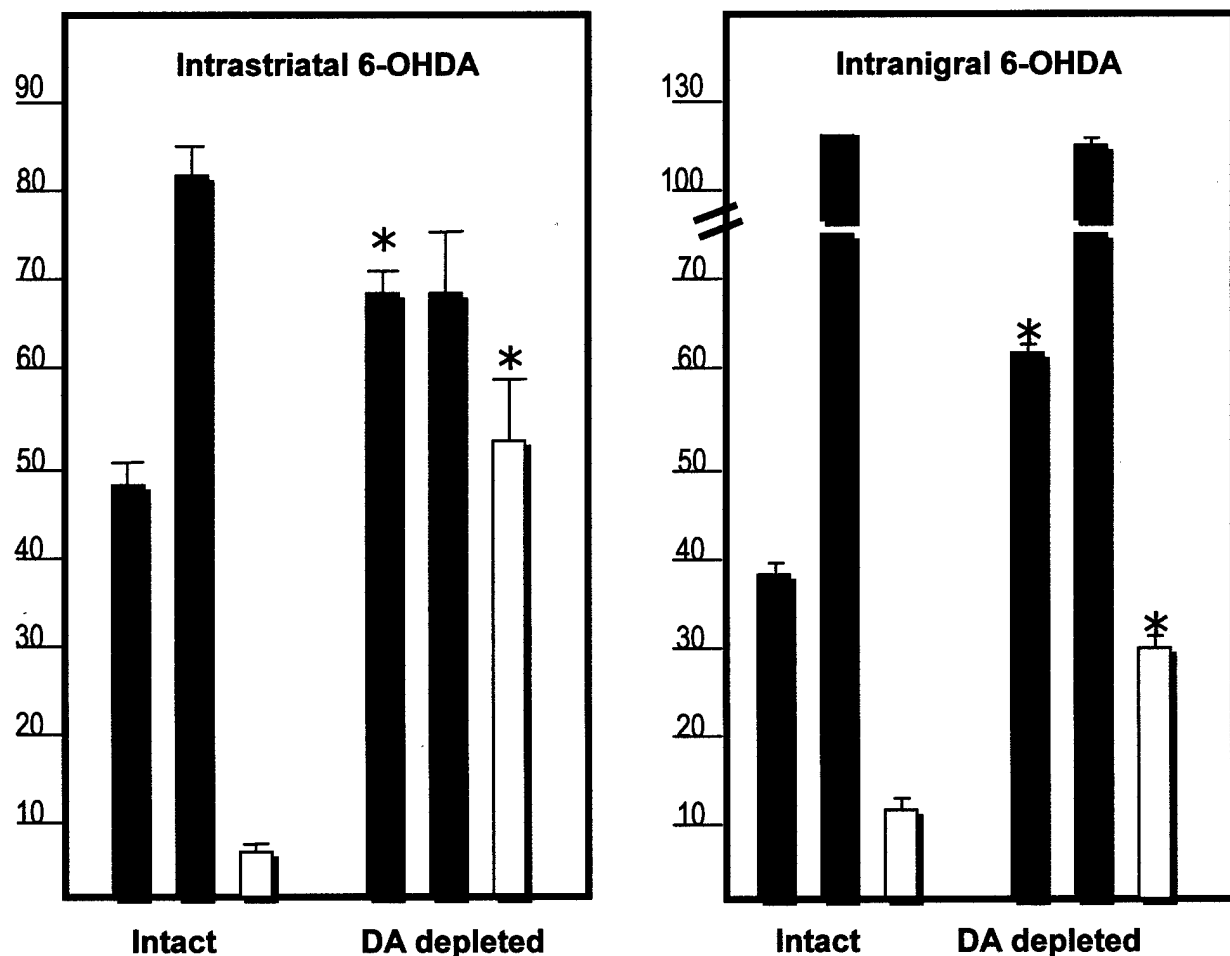
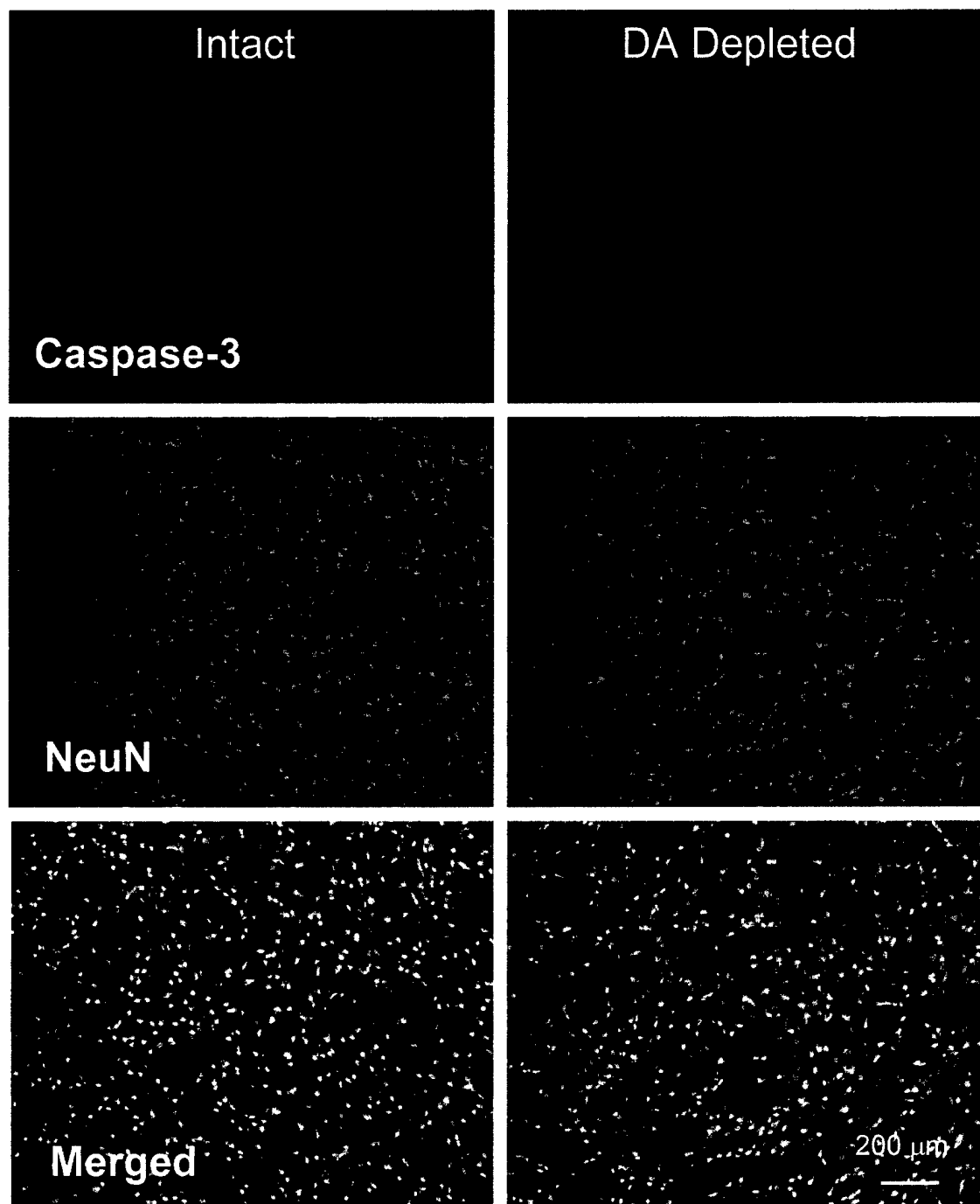


Figure 8. Rats were prepared as described above ($N = 4$), with unilateral intranigral 6-OHDA neurotoxin infusions, and survived 1 week to examine acute changes in striatal responses to sub-total DA depletion. The data showed that neuronal caspase-3 luminosities were not significantly different at 1 week following infusion of the neurotoxin, even though TH staining, indicative of DA nigrostriatal terminal boutons, was decreased $\sim 50\%$ (data not shown).



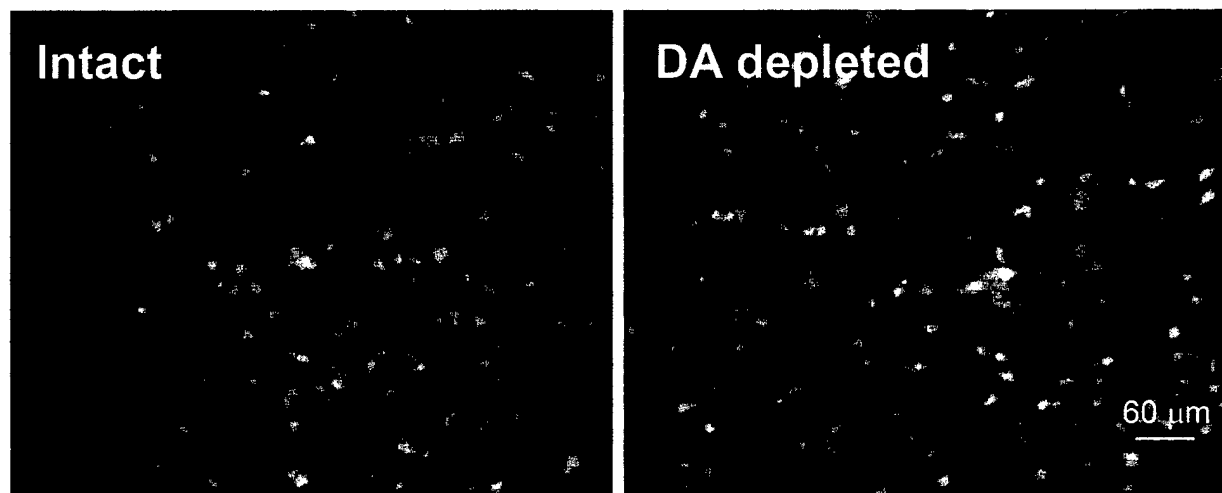
Preclinical PD rats that were lesioned intranigally by 3 μmole malonate showed similar losses in DA depletion (assessed using HPLC, figure 4) with corresponding elevations in neuronal cleaved caspase-3 within the ipsilateral striatum (data not shown), as rats lesioned using

intrastratial 6-OHDA infusion. We extended these studies to evaluate whether the enhanced caspase-3 activity could be associated with specific striatal projection pathways. First year medical school student, Thomas Buchanan, is completing this series of experiments as a summer fellowship. The data demonstrate significant elevations in enkephalinergic striatopallidal cleaved caspase-3 staining within the DA depleted striatum compared to the intact nucleus (intact: 95.38 ± 3.38 luminosity values; DA depleted: 117.35 ± 3.96 luminosity values; $p < 0.001$ $N=6$). There are 14 animals in this experimental group, but only six have been morphologically analyzed to date.

8-oxoguanine after DA Depletion

We hypothesized that formation of reactive oxygen species might be a potential cellular mechanism for the enhanced striatal caspase-3 levels following DA depletion. As an initial test of this theory, we examined the co-expression of 8-oxoguanine, a marker of oxidative DNA damage that has been associated with hypoxia (Lee et al, 2002), Alzheimer's disease (Lovell and Markesbery, 2001), aging (Hirano et al, 1996), and PD (Alam et al, 1997). We purchased antisera generated against 8-oxoguanine and double-stained sections of our preclinical PD rat model with cleaved caspase-3. This is shown for a representative experiment in figure 9.

Figure 9. The preclinical PD rat was produced by intranigral infusion of 3 μ mole malonate, and killed 4-weeks following the neurotoxin infusion. Cleaved caspase-3 was detected using Cy3 (red), while 8-oxoguanine was elucidated by Cy2 (green). The images were merged electronically to demonstrate cellular co-expression as a yellow signal. The number and signal luminosity for the activated caspase is elevated significantly ($p < 0.001$) following DA depletion (98.93 ± 3.3) compared to the intact striatum (73.45 ± 1.9). This also occurred for 8-oxoguanine, intact (107.98 ± 1.7) versus the DA depleted striatum (116.15 ± 2.3) @ $p < 0.001$.

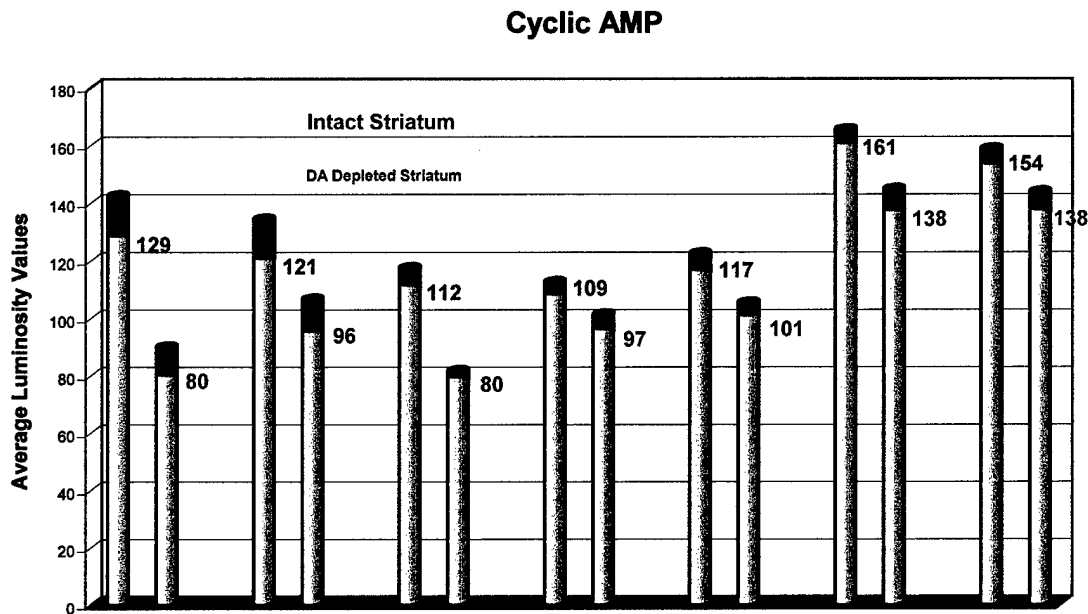


These animals are still undergoing morphological analyses, and this forms the basis for an independent study project for a college senior student intern, Anne E. Grissell. Portions of this work will be presented in poster form (Buchanan et al, 2002) at the forthcoming New York Academy of Sciences sponsored DoD Workshop on "Parkinson's Disease: The Life Cycle of the Dopamine Neuron," in Princeton, New Jersey (September 18-20, 2002).

Cyclic AMP and Phosphorylated NR1 NMDA Receptor Subunits

We have begun to examine the distribution and intensity of the cyclic AMP staining in the malonate lesioned, preclinical PD rats. J. Theobald, a first-year MD/Ph.D student, undertook this project. The cyclic AMP staining results were analogous to the observations made using intrastriatal 6-OHDA lesions to produce the early phase of PD, namely there was a consistent and statistically significant elevation in cyclic AMP ipsilateral to the neurotoxin infusion and subsequent DA depletion. This is shown in a representative animal in figure 10.

Figure 10. Cellular luminosity values were determined for individual neurons within the striatum, using methods described in Ariano et al (2002), and outlined above. The data presented are based upon 500 neurons in one experimental animal. The histogram shows the averaged luminosity (staining intensity) for regions of the dorsal striatum. The intact (blue and on the right of the pair) and DA depleted (red and to the left of each pair) striatal areas were compared using a two-tailed t-test, and considered significant at $p < 0.001$. All pairs achieved this level of significance. The sem of the luminosity is shown in green above the respective luminosity value.



A number of intracellular proteins are targets for cyclic AMP-mediated phosphorylation through the action of protein kinase A. Post-translational processing produces functional enhancements (and inhibitions) and is particularly important for recycling and targeting neurotransmitter receptors and channel ionophores to neuronal membrane regions undergoing heightened activity. As an initial survey of potential ionophore candidates that might be affected by the enhanced levels of cyclic AMP detected in the DA depleted striatum in our preclinical PD model, we evaluated the level and distribution of the obligatory subunit of the NMDA receptor, NR1. NMDA receptors are heteromeric ligand-gated ion channels that assemble in an unknown stoichiometry from the cloned subunits NR1, N2A – D and their splice variants (Seeburg, 1993; Hollmann and Heinemann, 1994). The NR1 subunit is expressed robustly within medium spiny projection neurons of the striatum (Standaert et al, 1999), and it is specifically phosphorylated by protein kinase A at serine residue 897 (Leonard and Hell, 1997). Cyclic AMP-mediated modification enhances the functioning of the NMDA receptor (Raman et al, 1996; Westphal et al,

1999) and changes its synaptic distribution (Hall and Soderling, 1997). Moreover, a growing body of experimental evidence suggests the involvement of glutamate over activity in the manifestations of DA depletion in models of PD (Zuddas et al, 1992; Emmi et al, 1996; Andres et al, 1998; Vila et al, 1999; Jonkers et al, 2000). Using site-specific protein-kinase A phosphorylated anti-NR1 antisera (Tingley et al, 1997), we can assess changes in the distribution and expression of the modified subunit.

Figure 11. Immunofluorescent detection of protein kinase A phosphorylated NR1 subunits 4 weeks after 3 μ mole intranigral malonate infusion. Cellular luminosity values for ~600 neurons were obtained and averaged by regional area of the dorsal striatum. Statistical comparisons used *t*-tests and attained significance at $p < 0.001$. The sem of the averaged luminosity values are shown in green above the appropriate intact (blue, left of each pair) or DA depleted (red, right of each pair) striatal region.

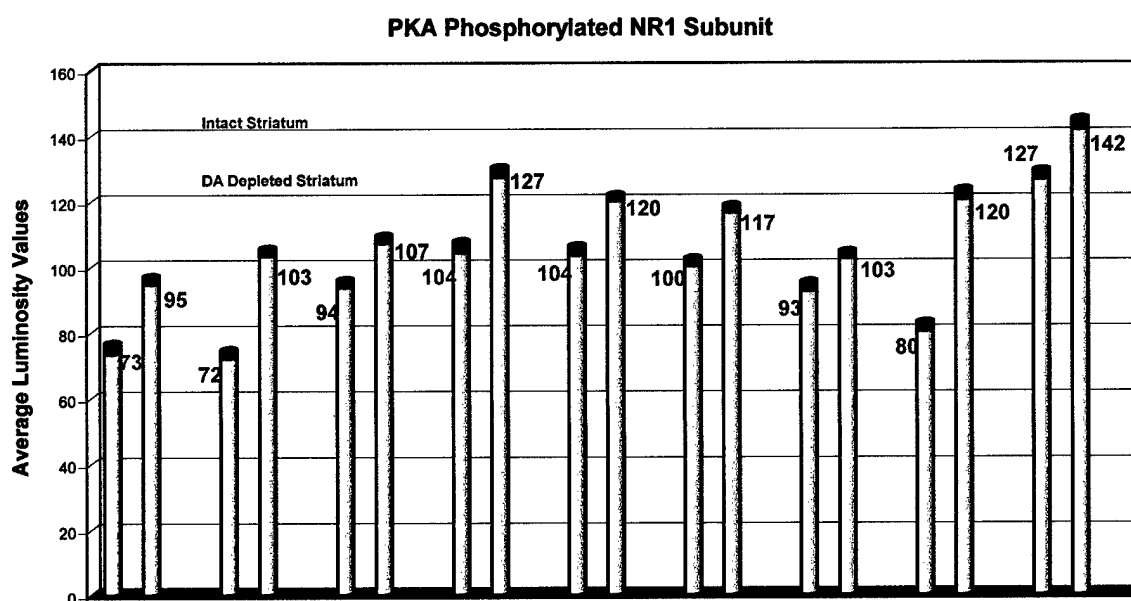


Figure 12. Immunofluorescent expression of the protein kinase A phosphorylated form of the NR1 subunit (at the ser897 site) of the NMDA receptor was detected using an affinity-purified rabbit-derived antisera. Image acquisition parameters were normalized to the intact side (left panel). Medium diameter neuron somata are stained robustly, as are their processes that travel throughout the striatal neuropil and give fluorescent staining intensity to this compartment when contrasted to the lack of signal in the myelinated fiber bundles of the internal capsule that penetrate the striatum. DA depletion (right panel), caused by intranigral infusion of 3 μ mole malonate produced a substantial striatal enhancement in expression of the phosphorylated subunit.



Key Research Accomplishments:

- The species relevant odorant stressor TMT used in a mild regimen of one hour-long exposure/week for 5 weeks, 4 weeks following intrastriatal 6-OHDA DA depletion produced an accelerated loss in striatal DA levels and its turnover (DOPAC:DA ratio). These results were statistically significant in comparison to non-TMT treated 6-OHDA-lesioned rats.
- Intranigral infusion of malonate caused a substantial acute behavioral response in all rats, regardless of the dose infused. Use of 3 μ mole intranigral malonate (in 1 μ l) provided the criteria of ~50% loss of striatal DA, 4 weeks post-infusion as assessed by HPLC.
- Rats lesioned with this dose of malonate demonstrated significant limb asymmetry in that the forepaw ipsilateral to the DA depletion was preferred for exploring a novel environment.
- Morphological assessment of intranigral malonate-lesioned rats was analogous to findings detected previously following intrastriatal 6-OHDA infusions. We detected ~50% loss in TH staining (as an index of presynaptic DA nigrostriatal terminals), elevations in the D2 DA receptor subtype, elevations in the number of cyclic AMP stained cells and their luminosity, and enhanced neuronal cleaved caspase-3 expression that were statistically significant.
- Extending the studies on cleaved caspase-3 showed that some of the elevated staining intensity was associated specifically with the indirect, enkephalinergic striatopallidal pathway.
- A potential cellular mechanism for the enhanced striatal caspase-3 levels in response to altered DA homeostasis may be due to formation of reactive oxygen species and subsequent oxidative damage of DNA. This theory is supported by our preliminary findings that 8-oxoguanine is elevated within the DA depleted striatum and co-expressed with neuronal cleaved caspase-3 staining.
- The elevations in cyclic AMP detected in the DA depleted striatum will alter downstream protein kinase A signaling. We have found that the protein kinase A phosphorylated form of the NR1 subunit of the NMDA receptor is elevated significantly, ipsilateral to the intranigral malonate infusion.

Yet to be Accomplished:

1. We need to prepare a series of intranigral malonate infused rats, and expose them to the secondary stressor paradigm using TMT. This will be one of the first tasks investigated this fall. These rats will be evaluated by HPLC, morphology, and behavioral analyses and the results conjoined.
2. We have a series of 6-OHDA lesioned animals that have been exposed to a more aggressive TMT regimen (3 times/week), which were behaviorally videotaped for limb asymmetries. These data need to be scored. The results will be correlated with the placement of the neurotoxin, as half of the animals received intra~~striatal~~ infusions of 6-OHDA, while the rest received intra~~nigral~~ infusion of 6-OHDA.
3. The brains from these rats (in #2) are awaiting morphological evaluation, and will be processed for DA receptor subtype expression, cyclic AMP staining, cleaved caspase-3, phosphorylated NR1 within defined striatal projection systems.
4. Western analyses needs to be attempted on the frozen/thawed/refrozen samples that were prepared in the previous year. We will examine D2 DA receptor, cyclic AMP, cleaved caspase-3, and 8-oxoguanine levels on these tissues once the methods are established.

Reportable Findings/Presentations

- Littlejohn FC, Ariano MA (2002) Caspase-3 activity is increased in striatal neurons after subtotal 6-OHDA nigrostriatal lesions. *Chicago Soc Neuroscience Chapter*.
- Buchanan T, Grissell AE, Ariano MA (2002) Striatopallidal changes in a preclinical rat model of Parkinson's disease. *Ann NY Acad Sci*.
- Ariano MA, Littlejohn FC, Buchanan T, Collier KS (2002) Neurochemical and behavioral changes in a preclinical rat model of Parkinson's disease. *Soc Neurosci* 28.

Conclusions

The partially DA depleted rat provides a credible model of preclinical stages of PD. The animals exhibit a subtle behavior correlated to the DA imbalance, which can be evaluated prior to sacrifice and used to detect further deterioration of the nigrostriatal pathway. Morphological correlates of the HPLC-determined partial DA depletion include consistent elevations in the D2 DA receptor protein, cyclic AMP second messenger, and the activated form of caspase-3. These changes are significant, and are unexpected in light of the level (~50%) and duration (1 month) of the DA loss. These findings suggest substantial postsynaptic (striatal) changes occur in the early stages of PD, and provide a mechanism to evaluate initial neurochemical alterations that may be modified to retard or stop the insidious progress of PD. Another surprising outcome is our observation that a weekly exposure to an odorant stressor produced a profound additional loss in the levels of striatal DA and its turnover. These data argue strongly that stress will enhance the onset and subsequent development of PD. Finally, our preliminary investigations of target candidates downstream in the cyclic AMP signaling pathway provide an alternative avenue to intervene in the pharmacotherapy of PD. The finding that a subunit of the NMDA receptor is modified selectively and enhanced in its expression following partial DA loss demonstrates the complexity of PD and cautions our thinking of the disorder as principally dopaminergic in scope.

In closing, we need to bolster our animal group numbers in order to present this work for peer-review and publication. This is a very high priority in the next few months.

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Appendix Materials

ASSISTANCE AGREEMENT

2303

AWARD TYPE: <input checked="" type="checkbox"/> GRANT (31 USC 6304) <input type="checkbox"/> OPERATIVE AGREEMENT (31 USC 6305) <input type="checkbox"/> OTHER TRANSACTION (10 USC 2371)			
AWARD NO: DAMD17-99-1-9542 Modification P00002	EFFECTIVE DATE See Grants Officer Signature Date Below	AWARD AMOUNT \$414,198.00	Page 1 of 1 Wendy A. Cockerham 301-619-2034/phone 301-619-4084/fax
PROJECT TITLE: "Degenerative Risks for Parkinson's Disease after Toxin Exposure and Stress" <div style="text-align: right;">CFDA 12.420</div>			
PERFORMANCE PERIOD: 01 Jul 1999 - 01 Feb 2004 (research ends 01 Jan 2004)		PRINCIPAL INVESTIGATOR: Dr. Marjorie Ariano	
AWARDED AND ADMINISTERED BY: U.S. Army Medical Research Acquisition Activity ATTN: MCMR-AAA-A 820 Chandler St. Fort Detrick Maryland 21702-5014		PAYMENTS WILL BE MADE BY: EFT:T Army Vendor Pay DFAS-SA/FPA 500 McCullough Avenue San Antonio, TX 78215-2100	
DUNS No: 01-721-1439 TIN No:		(SEE PARAGRAPH TITLED "PAYMENTS" FOR INSTRUCTIONS)	
AWARDED TO: Finch University of the Health Sciences The Chicago Medical School 3333 Green Bay Road North Chicago, IL 60064-3095		REMIT PAYMENT TO: Finch University of the Health Sciences The Chicago Medical School 3333 Green Bay Road North Chicago, IL 60064-3095	
ACCOUNTING AND APPROPRIATION DATA: not applicable			
SCOPE OF WORK: A. The purpose of this modification is to extend the period of performance to read as shown above. This is being done at no additional cost to the Government and in accordance with the recipient's request dated 28 March 2002. The period of performance is hereby changed FROM: 01 July 1999 - 01 August 2002 (research ends 01 July 2002) TO: 01 July 1999 - 01 February 2004 (research ends 01 January 2004) B. All other terms and conditions remain unchanged.			
RECIPIENT		GRANTS OFFICER	
ACCEPTED BY: signature not required in accordance with recipient's letter dated 3/28/02 <hr style="width: 30%; margin-left: 0;"/>		UNITED STATES OF AMERICA <div style="text-align: center;"> <hr style="width: 80%; margin: 0 auto;"/> </div>	
SIGNATURE		SIGNATURE	
NAME AND TITLE	DATE	NAME AND TITLE	DATE
		Cheryl R. Miles GRANTS OFFICER	5/20/02



28 March 2002

Ms. Wendy Cockerham
Contract Specialist
US Army Medical Research
MCMR-AAA, NETRP
820 Chandler Street
Fort Detrick, MD 21702-5014

DAMD 17-99-1-9542

Dear Ms. Cockerham,

I am requesting a no-cost extension for my proposal entitled, "Degenerative Risks for Parkinson's Disease after Toxin Exposure and Stress." I would ask that the Army extend the duration of the project for 18 months, and my rationale for this extension is provided below. In addition, I propose a change in the SOW to reflect the alteration in duration and scope of the project.

- **Personnel issues.** In the course of the past two and one half years we have had a very difficult time identifying appropriate laboratory technical help. This has substantially slowed the progress of the present award, as the day-to-day running of the lab has been inconsistent without this type of individual on-board. A new research assistant was hired in August 2001. As a consequence, large numbers of animals are infused with neurotoxin at a single time, using the technical expertise of two colleagues at Rush-St. Luke's Presbyterian Medical Center in Chicago (Dr. T.J. Collier and Mr. B. Daley). This has enabled us to evaluate the neurotoxin effects on nigrostriatal functioning much more thoroughly, although there is still a "bottle-neck" in completing our morphological analyses. It has become apparent that I must perform this analysis, as the research assistant does not have the background to make judgments on the morphological outcomes of the experiments. I am not always available to facilitate this aspect of the research due to other commitments and responsibilities. This will necessitate increasing the duration of the research program to complete these morphological studies. Because we experienced gaps in our staff employment, we have sufficient funds to cover the salary of a ¾ time lab assistant through the next year.
- **Departure of the co-PI.** As of 15 April 2002, the co-PI on the grant, Dr. Kathy Collier will be leaving the Chicago Medical School. Dr. Collier will not receive any further salary compensation from this point forward, providing additional funds to support continued technical assistance for the project. Dr. Collier will be relocating to Rush-St. Luke's Presbyterian Medical Center in Chicago, and thus will still be able to provide input to the scientific scope of the project.



- **Freezer debacle.** During the weekend of December 8-9, 2001, the electrical circuit breaker on the generator-backed up power line that serves my -85°C freezer was tripped, terminating electricity to the unit. The loss of power was not reported to me during the weekend, and thus was not discovered until I arrived at work on Monday morning. The internal temperature of the freezer was at 0°C by that point, and the inside was dripping with moisture. There were 26 freezer towers with 7-9 boxes containing custom produced antisera, peptides, and animal brain tissues accumulated over the past 20 years. Four towers contained brain tissue prepared for the DoD neurotoxin program 02 year studies, which were lesioned and had been exposed to a predator odorant as a secondary stressor event (as well as an experimental transgenic Huntington's disease brain bank). The brain tissues did not survive the increase in temperature, and cannot be used for the planned morphological analyses. We are thus re-preparing this tissue for the current proposal in addition to the planned 03 year complement of experiments, and this will take additional time to complete. We have retained the thawed and refrozen samples, and are in the process of assessing whether or not they are viable for biochemical analyses, such as western determination of protein levels. The University has provided monies to directly replace the cost of animals and their per diem, but this hardly covers the loss of nearly 1 year of research, and the stunning setback in time and morale.

These three reasons therefore, have motivated this request for an 18-month extension to complete the proposed investigations of Parkinson's disease and secondary stressor events.

This will necessitate a change in the SOW since the time frame has been altered considerably. I would propose the following measures, detailed below.

We had approximately 40 brains from the experimental paradigms used in the 02 year of our research program that no longer have sufficient integrity to analyze morphologically, I propose that we attempt to perform western assays for dopamine receptor levels on that tissue. We have begun to establish the appropriate protocols for this purpose using our D1 dopamine receptor specific antisera, and the results look promising. I anticipate we also will be able to establish protein levels for the D2 receptor in these tissues, since both D1 and D2 receptor proteins exist in sufficient quantity to detect in whole striatal homogenates. However, I do not think it is feasible to expect consistent detection for the D3, D4, or D5 dopamine receptor proteins due to low protein levels in a full striatal homogenate. We will at least attempt this method on intact rats before abandoning the approach. If we cannot detect D3, D4, and D5 dopamine receptors using westerns, we will re-prepare the 02 year brains for sufficient morphological evaluations to provide statistical confidence using luminosity measurements and qualitative trends in the results.

Our survey to evaluate other potential cellular mechanisms underlying postsynaptic changes in the nigrostriatal pathway following neurotoxin exposure has elucidated some intriguing preliminary data. We have demonstrated that infusion of the neurotoxin 6-OHDA into the striatum, to yield a subtotal dopamine depletion lesion ($6\text{ }\mu\text{gm}$ in $2\text{ }\mu\text{l}$ into the striatum, as described in the 01 and 02 year progress reports), produced an elevation in the active form (e.g., cleaved) pre-



apoptotic enzyme, caspase-3, at four weeks after the neurotoxin lesion ($N = 4$ rats). This was most intriguing, as it suggested to us that a mild insult to the dopamine afferents would predispose the striatal neurons, which are the primary targets of drug therapy in Parkinson's disease, to cellular malfunction and eventual degeneration. A potential confound of our paradigm was that infusion of the neurotoxin could substantially damage the parenchyma of the striatum, and that in turn could predispose the striatal cells to apoptosis. We have extended our investigation of this phenomenon, and determined that infusion of 6-OHDA into the substantia nigra ($6 \mu\text{gm}/2 \mu\text{l}$) also produces the same elevation in cleaved caspase-3 in the postsynaptic striatum at the four week time point ($N = 4$ animals). We further determined that the elevation of the cleaved caspase-3 occurred in neurons, using the simultaneous detection of NeuN, a neuron-specific marker ($N = 4$ animals). Further, it appears that the elevation of caspase may be associated specifically with the enkephalin producing, indirect striatopallidal system, which demonstrates enhanced functioning following losses of nigrostriatal dopamine ($N = 2$ animals). We propose to pursue these investigations in the extended grant period and evaluate the time course of this change, and if other markers of apoptosis or oxidative stress occur in particular striatal neuron populations. We propose to examine cytochrome oxidase complex 1 (COX1) and 8-oxoguanine (a marker of reactive oxygen species damage to DNA) in these studies following unilateral 6-OHDA nigral infusions. These studies will be assessed using double immunofluorescence staining and we also will attempt to analyze the changes quantitatively using western analyses.

We need to reproduce the secondary stressor experiments following neurotoxin infusions using both 6-OHDA and malonate. We propose to establish a more frequent exposure regimen as our preliminary HPLC findings suggested that one hour per week exposure to the predator odor, TMT was insufficient to produce changes in striatal or nigral dopamine levels or turnover, even though the animals exhibited robust behavioral responses to its presentation. We will evaluate these experiments using HPLC, dopamine receptor and cyclic AMP immunofluorescence in the striatum, and specific expression of cleaved caspase-3 staining in the enkephalin striatopallidal pathway using double immunofluorescence techniques.

We have begun to video tape the behavior of our neurotoxin lesioned animals, using the forepaw placement rating described by Dr. T. Schallert (Tillerson et al, *J Neurosci* 21:4427, 2001). We are hopeful this will demonstrate subtle behavioral changes that occur with the subtotal nigrostriatal dopamine lesions to enable us to relate the biochemical and morphological differences with a phenotype.

There are potential problems that might preclude the successful completion and performance of these proposed experimental approaches. The most obvious confound is the low level of protein expression for the D3, D4, and D5 dopamine receptors, which may preclude their detection using western analysis. We will need to examine this in the upcoming months. We are still evaluating the appropriate TMT exposure regimen that will initiate a biochemical and morphological change in the nigrostriatal system, without habituating the animals to the stressor event. I am sure there are other considerations we have not encountered that could impact on the success-



Finch University of Health Sciences - The Chicago Medical School

Marjorie A. Ariano, Ph.D.
Professor of Neuroscience

ful performance of these renewed aims. We will re-evaluate our paradigms as we encounter them, and keep the DoD apprised of our progress.

Thank you for your time and attention. If there are other items of information that you need, please contact me by telephone at (847) 578-3412, fax (847) 578-8515, or by email using arianom@finchcms.edu.

Sincerely,

Marjorie A. Ariano, Ph.D.
Professor, and PI of the project

Velayudhan Nair, Ph.D, D.Sc
VP of Research and
Dean School of Graduate and
Postdoctoral Studies